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POLYSACCHARIDE VACCINE FOR STAPHYLOCOCCAL INFECTIONS

Related Applications

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 60/425,425, filed November 12, 2002, and entitled "POLYSACCHARIDE VACCINE FOR STAPHYLOCOCCAL INFECTIONS", the contents of which are herein incorporated by reference in their entirety.

Government Support

The present invention was supported in part by a grant from the United States National Institutes of Health AI46706. The U.S. Government may retain certain rights in the invention.

Field of the Invention

The present invention relates to polysaccharide compositions useful for inducing immunity for the prevention and treatment of *Staphylococcal* infections. The invention also relates to methods of making and using polysaccharide based antigens, related antibodies and diagnostic kits and for inducing active and passive immunity using the polysaccharide material and antibodies thereto.

Background of the Invention

Staphylococci are gram-positive bacteria which normally inhabit and colonize the skin and mucus membranes of humans. If the skin or mucus membrane becomes damaged during surgery or other trauma, the *Staphylococci* may gain access to internal tissues causing infection to develop. If the *Staphylococci* proliferate locally or enter the lymphatic or blood system, serious infectious complications such as those associated with *Staphylococcal* bacteremia may result. These complications include septic shock, endocarditis, arthritis, osteomyelitis, pneumonia, and abscesses in various organs.

Staphylococci include both coagulase-positive organisms that produce a free coagulase and coagulase-negative organisms that do not produce this free coagulase. *Staphylococcus aureus* is the most common coagulase-positive form of *Staphylococci*. *S.*

aureus generally causes infection at a local site, either extravascular or intravascular, which ultimately may result in bacteremia. *S. aureus* is also a leading cause of acute osteomyelitis, and causes *Staphylococcal* pneumonia infections. Additionally, *S. aureus* is responsible for approximately 1-9% of the cases of bacterial meningitis and 10-15% of brain abscesses.

There are at least twenty-one known species of coagulase-negative *Staphylococci*, including *S. epidermidis, S. saprophyticus, S. hominis, S. warneri, S. haemolyticus, S. saprophiticus, S. cohnii, S. xylosus, S. simulans,* and *S. capitis*. *S. epidermidis* is the most frequent infection-causing agent associated with intravenous access devices, and the most frequent isolate in primary nosocomial bacteremias. *S. epidermidis* is also associated with prosthetic valve endocarditis.

Staphylococcus is also a common source of bacterial infection in animals. For instance, *Staphylococcal* mastitis is a common problem in ruminants such as cattle, sheep, and goats. The disease is generally treated with antibiotics to reduce the infection but the treatment is a costly procedure and still results in a loss of milk production. The most effective vaccines identified to date are live, intact *S. aureus* vaccines administered subcutaneously. The administration of live vaccines, however, is associated with the risk of infection. For that reason, many researchers have attempted to produce killed *S. aureus* vaccines and/or to isolate capsular polysaccharides or cell wall components which will induce immunity to *S. aureus*. None of these attempts, however, has been successful.

Summary of the Invention

The present invention relates to methods and products useful for immunization of humans and animals against infection by coagulase-negative and coagulase-positive *Staphylococci*. It has been discovered, according to the invention, that a poly N-acetyl glucosamine (PNAG) surface polysaccharide from *Staphylococci,* such as *S. aureus* and *S. epidermis*, that is poorly substituted with acetate residues, is highly immunogenic *in vivo* and preferentially elicits antibodies that mediate opsonic killing and protection from infection. This polysaccharide is therefore useful, inter alia, in the generation of immune responses, including antibody dependent immune responses, to *Staphylococci*.

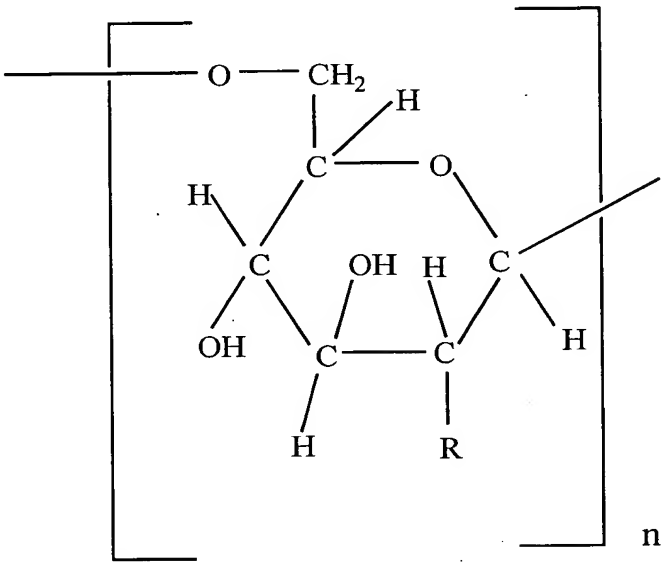
In one aspect, the invention provides a composition comprising an isolated polysaccharide comprising a β-1,6-glucosamine polymer, having a length of at least two monomeric units, wherein less than 50% of glucosamine amino groups are substituted with

acetate. In one aspect, the composition is sterile (e.g., it would be suitable for in vivo injection). In another aspect, , the invention provides a composition comprising an isolated polysaccharide comprising a β-1,6-glucosamine polymer, having a length of at least two monomeric units, wherein less than 50% of glucosamine amino groups are substituted with acetate and wherein the polysaccharide is conjugated to a carrier compound.

As used throughout, "a polysaccharide of the invention" refers to *Staphylococcal* poly-N-acetyl glucosamine (PNAG) surface polysaccharide having less than 50% acetate substitutions. This polysaccharide is referred to herein as deacetylated PNAG (dPNAG). It is to be understood that dPNAG may be wholly or partially deacetylated, provided that the range of acetylation is from 0 to less than 50%. As used herein, native PNAG is a mixture of PNAG forms with varying degrees of acetylation. Native PNAG may include dPNAG, however it is present in a mixture with highly acetylated forms of PNAG. As used herein, a "highly acetylated" form of PNAG is a PNAG having greater than 50% acetate substitutions.

Several embodiments apply equally to the various aspects of the invention. These embodiments are recited below.

In one embodiment, the isolated polysaccharide is defined by the following structure:



wherein n is an integer greater than or equal to four, R is selected from the group consisting of -NH-CO-CH_3 and -NH_2, and less than 50% of the R groups are -NH-CO-CH_3. According to some aspects of the invention in which the polysaccharide is conjugated to a carrier compound or a linker joined to a carrier compound, n can be 2, 3, 4 or greater.

In one embodiment, the polysaccharide has a molecular weight of at least 800 Daltons, while in other embodiments, the molecular weight is at least 1000 Daltons. In still further embodiments, the molecular weight is selected from the group consisting of at least 1200 Daltons, at least greater than 2000 Daltons, at least 2500 Daltons, at least 5000 Daltons, at least 7500 Daltons, at least 10,000 Daltons, at least 25,000 Daltons, at least 50,000 Daltons, at least 75,000 Daltons, and at least 100,000 Daltons. In still further embodiments, the molecular weight is selected from the group consisting of at least 125,000 Daltons, at least 150,000 Daltons, at least 200,000 Daltons, at least 250,000 Dalton, at least 300,000 Daltons, at least 350,000 Daltons, at least 400,000 Daltons, at least 450,000 Daltons, and at least 500,000 Daltons.

The isolated polysaccharide may have a length of at least two, at least three, at least four, at least five, or at least six monomeric units. In other embodiments, the length of the polysaccharide is selected from the group consisting of at least 6, at least 10, at least 20, at least 50, at least 100, at least 200, at least 300, at least 400, and at least 500 monomer units.

In other embodiments, equal to or less than 45%, equal to or less than 40%, equal to or less than 35%, equal to or less than 30%, equal to or less than 25%, equal to or less than 20%, equal to or less than 15%, equal to or less than 10%, equal to or less than 5%, or equal to or less than 1% of glucosamine amino groups (or R groups) are substituted with acetate. In still other embodiments, none of the glucosamine amino groups is substituted with acetate. The dPNAG may refer to any of these.

Accordingly, the polysaccharide may be a hetero-substituted polymer, wherein the R groups are a mixture of acetate substitutions (i.e., -NH-CO-CH_3) and unsubstituted amine (i.e., -NH_2) groups, provided that less than 50% of these groups are substituted with acetate. The polysaccharide can also be homo-substituted if all of the R groups are amines (i.e., none is acetate-substituted).

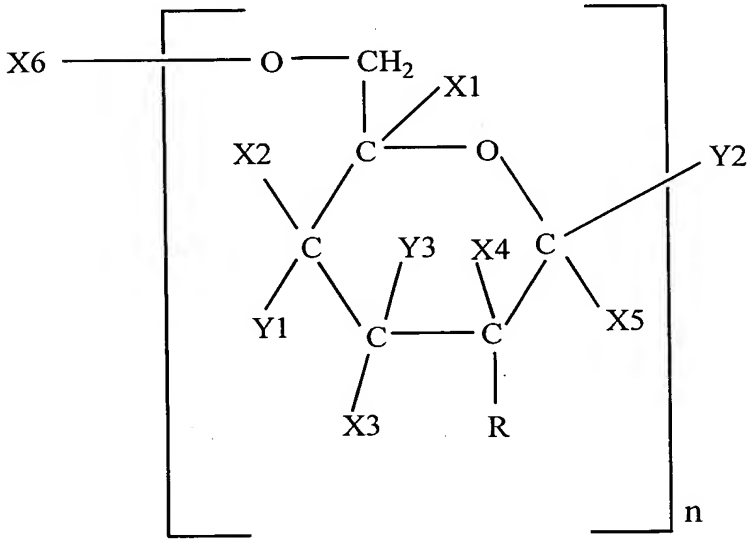
In some embodiments of the invention, the isolated polysaccharide may be conjugated to a carrier compound. The carrier compound may be conjugated to the polysaccharide via a linker. The carrier compound may be a peptide carrier, but it is not so limited.

In these and other embodiments, the composition comprising the isolated polysaccharide may further comprise a pharmaceutically acceptable carrier.

In some embodiments, the composition is at least 90% pure, at least 95% pure, at least 97% pure, or at least 99% pure (i.e., at least 90%, at least 95%, at least 97% or at least 99% of

the polysaccharide present in the composition is dPNAG). In yet other embodiments, the composition is substantially free of phosphate or teichoic acid. Preferably, the composition is substantially free of polysaccharides having greater than 50%, greater than 75%, or greater than 90% acetate substitution at the glucosamine amino (R) group.

In some embodiments, the polysaccharide consists of the following structure:



wherein each of X1, X2, X3, X4, X5 and X6 is either H, a carrier compound, or a linker joined to a carrier compound; and each of Y1, Y2 and Y3 is either OH, a carrier compound, or a linker joined to a carrier compound. In some embodiments, only one carrier compound or linker joined to a carrier compound is conjugated to the structure. In other embodiments, only one of X1, X2, X3, X4, X5 or X6 is conjugated to a carrier compound or a linker joined to a carrier compound. In still other embodiments, only one of Y1, Y2 or Y3 is conjugated to a carrier compound or linker joined to a carrier compound. In still other embodiments, the carrier compound or linker conjugated thereto is conjugated at only one of the X1, X2, X3, X4, X5, X6, Y1, Y2 or Y3 positions. The carrier compound may be a polysaccharide. In other embodiments, the carrier molecule is a polysaccharide optionally substituted directly, or through a linker, with one or more carrier compounds, such as other polysaccharides, peptides, and the like. In some embodiments, the carrier polysaccharide is not an N-acetyl beta (β) 1-6 glucosamine. According to some aspects of the invention in which X is a carrier compound or a linker joined to a carrier compound, n can be 2, 3, 4 or greater.

The invention provides pharmaceutical compositions comprising any of the polysaccharides of the invention, which may be used as vaccines. These compositions comprise the polysaccharide in an amount effective to stimulate an immune response, such as an antigen-specific immune response. The vaccine composition may further comprise a pharmaceutically acceptable carrier and/or an adjuvant. The pharmaceutical composition may contain the polysaccharide conjugated to a carrier compound, either directly or through a linker.

Other aspects of the invention provide methods for making the polysaccharides of the invention. These methods are described below.

In one aspect, the invention provides an isolated polysaccharide prepared according to the following method: ethanol precipitating a crude polysaccharide preparation from a concentrated bacterial cell body preparation; concurrently digesting the crude polysaccharide with lysozyme and lysostaphin followed by sequential digestion with a nuclease and proteinase K to form a digested polysaccharide preparation; size fractionating the digested polysaccharide preparation; isolating an acetylated polysaccharide fraction; and de-acetylating the acetylated polysaccharide to produce a deacetylated polysaccharide (i.e., a polysaccharide having less than 50% acetate substitution).

In another aspect, the invention also provides a polysaccharide antigen comprising a polysaccharide prepared according to the following method: preparing an impure polysaccharide from a bacterial culture; incubating the impure polysaccharide with an acid or a base to produce a semi-pure polysaccharide; neutralizing the preparation; and incubating the neutralized preparation in hydrofluoric acid. In one embodiment, the method further involves isolating an acetylated polysaccharide from the preparation, and de-acetylating the acetylated polysaccharide to produce a deacetylated polysaccharide. In one embodiment, the acetylated polysaccharide is chemically de-acetylated, to a desired degree that is less than 50%. In another embodiment, the acetylated polysaccharide is de-acetylated by incubation with a basic solution, to a desired degree that is less than 50%. In still another embodiment, the acetylated polysaccharide is enzymatically de-acetylated.

Various embodiments apply to the foregoing methods. Some of these additional embodiments are recited below. The bacterial culture may be a coagulase-negative or a coagulase-positive *Staphylococcus* culture. The bacterial culture may be a *Staphylococcus*

aureus culture or a *Staphylococcus epidermidis* culture. In another embodiment, the polysaccharide preparation is size fractionated using a column.

An example of a preparation of the polysaccharide of the invention is as follows: A bacterial culture is incubated with a strong base or a strong acid to make an acid or a base solution. The acid or base solution is then neutralized to pH 2 to produce a crude antigen suspension. The crude antigen suspension is dialyzed against a solution such as deionized water, and insoluble crude antigen is collected. The insoluble crude antigen can be lyophilized and then resuspended in a buffer. The buffer can be selected from the group consisting of 50 mM PBS and 100 mM Tris with 150 mM NaCl. The strong base or acid can be greater than 1 N NaOH or 1 M HCl. In some embodiments, the strong base or acid is 5 N NaOH or 5 M HCl. In another embodiment, the bacterial culture extract is stirred in a strong base or acid for 18-24 hours. The strong base or acid extraction may be repeated. The method further involves treating the antigen preparation to remove amino-linked acetate groups until a desired degree of acetate substitution is reached, thereby producing the deacetylated PNAG. De-acetylation can be effected either chemically or enzymatically. As an example, the antigen preparation can be incubated at 37°C for 2-20 hours in 1.0 N NaOH. The incubation can also be performed in weaker basis for longer times or at higher temperatures or in stronger bases for shorter times or at lower temperatures.

The foregoing methods can alternatively involve isolating a fraction from the preparation having less than 50% acetate substitutions, without the need for additional deacetylation.

The invention, in yet another aspect, provides methods for making pharmaceutical compositions. In one embodiment, the polysaccharide is combined with a pharmaceutically acceptable carrier and/or adjuvant. In another embodiment, the polysaccharide is conjugated to a carrier compound, either directly or through a linker, and then optionally combined with a pharmaceutically acceptable carrier and/or an adjuvant.

Any of the deacetylated polysaccharides described herein (i.e., dPNAG) can be used in the therapeutic or prophylactic methods of the invention.

In another aspect, the invention provides a method for preventing a *Staphylococcus* infection in a subject, preferably a non-rodent subject. The invention involves administering to a subject in need thereof an effective amount for inducing an immune response against *Staphylococcus* of any of the polysaccharides of the invention. In some embodiments the

Staphylococcus is *Staphylococcus aureus,* and in others the *Staphylococcus* is *Staphylococcus epidermidis*.

The subject is any subject that can be infected with *Staphylococcus* and preferably is not a rodent. In some embodiments, the subject is a human subject, and in other embodiments the subject is a primate, horse, cow, swine, goat, sheep, dog or cat.

In some embodiments, the subject is at risk of exposure to *Staphylococcus,* and in other embodiments, the subject has been exposed to *Staphylococcus*. In some embodiments, the subject is a human over 60 years of age. The subject may be one that is healthy. In some embodiments, the subject has not received a medical device implant.

Preferably, the polysaccharide is formulated as a vaccine, as described herein or as is known in the art. In a related embodiment, the polysaccharide is administered with an adjuvant. In other embodiments, the polysaccharide is administered systemically to the subject. The antigen may conjugated to a carrier compound. In some embodiments, the carrier compound is a peptide carrier although it is not so limited.

In another aspect, the invention provides a method for inducing active immunity to a *Staphylococcal* infection in a subject. The method includes the step of administering to a subject an effective amount for inducing active immunity to a *Staphylococcal* infection of any of the foregoing polysaccharide-containing compositions. In one embodiment, the method is a method for inducing immunity to infection by *Staphylococcus aureus.* In another embodiment, the method is a method for inducing immunity to infection by *Staphylococcus epidermidis.*

A method for producing polyclonal or monoclonal antibodies is provided according to another aspect of the invention. The method involves administering to a subject an adjuvant and any of the polysaccharides of the invention in an effective amount for producing antibodies specific for *Staphylococcus,* and isolating antibodies from the subject. In these as well as other aspects of the invention, the polysaccharide is used as an antigen. In one embodiment the subject is human, while in others the subject is a non-human subject such as a rabbit, mouse or rat. The method may further comprise purifying the antibody.

In another aspect, the invention provides a method for generating monoclonal antibodies comprising administering to a subject an effective amount, for producing antibodies specific for *Staphylococcus,* of an isolated polysaccharide of the invention, and an

adjuvant, harvesting spleen cells from the subject, fusing spleen cells from the subject to myeloma cells, and harvesting antibody production from a fusion subclone.

According to yet another aspect of the invention, a method is provided for identifying a monoclonal antibody specific for a polysaccharide of the invention. The method involves inducing an immune response to the antigen in a non-human subject, isolating antibody producing cells from the subject, producing immortalized cells from the antibody producing cells, and testing the ability of the immortalized cells to produce the monoclonal antibody using a polysaccharide of the invention. The method, in one embodiment, also includes the step of isolating a monoclonal antibody from the supernatant of the immortalized cells.

The invention further provides a composition comprising an isolated binding agent that binds selectively to an isolated polysaccharide of the invention. In one embodiment, the isolated binding agent is a peptide. The peptide maybe an antibody, or a fragment thereof. The antibody may be a polyclonal antibody. The antibody may be a humanized antibody or a chimeric antibody. In some important embodiments, the antibody is a human antibody. In some embodiments, the isolated binding agent binds specifically to dPNAG. In other embodiments, the isolated binding agent binds to both dPNAG and highly acetylated forms of PNAG.

In some embodiments, the isolated binding agent is conjugated to a detectable label. The detectable label may be selected from the group consisting of a radioactive label, an enzyme, a biotin molecule, an avidin molecule or a fluorochrome. The isolated binding agent may be conjugated to a bactericide, such as an antibiotic.

According to another aspect of the invention, a method is provided for inducing passive immunity to *Staphylococcus* infection in a subject. The infection may be a *Staphylococcus aureus* infection or a *Staphylococcus epidermis* infection, but is not so limited. The method includes the step of administering to a subject an effective amount, for inducing opsonization of *Staphylococcus,* of one of the foregoing antibodies that bind to dPNAG.

The foregoing methods intended for prevention of a *Staphylococcal* infection can be performed on subjects at risk of developing such an infection. These methods can similarly be applied to the treatment of subjects having a *Staphylococcal* infection. The prophylactic and therapeutic methods of the invention can be used in subjects having or at risk of having an infection from a bacterial species that expresses native PNAG.

In a further aspect, the invention provides a method for treating a subject having a *Staphylococcus* infection comprising administering an isolated binding agent that binds to an isolated polysaccharide of the invention to a subject in an amount effective to inhibit the *Staphylococcus* infection. In important embodiments, the binding agent binds to highly acetylated forms of PNAG as well as dPNAG.

In one embodiment, the *Staphylococcus* infection is selected from the group consisting of *Staphylococcus epidermidis* infection and *Staphylococcus aureus* infection. In another embodiment, the isolated binding agent is conjugated to a bactericide, such as an antibiotic.

Another aspect of the invention provides a method for evaluating the ability of a polysaccharide to protect against *Staphylococcal* infection in a subject. The method involves administering to the subject an effective amount of the polysaccharide, wherein the polysaccharide induces active immunity, exposing the subject to a *Staphylococcus*, and testing for the presence of *Staphylococcus* in the subject.

In yet another aspect, the invention provides a method for identifying the presence of dPNAG in a sample, comprising contacting a sample with an isolated binding agent that binds to dPNAG; and detecting binding of the isolated binding agent to the sample. Binding of the isolated binding agent to the sample indicates the presence of dPNAG in the sample. If the binding agent also binds PNAG, then the method can also be used to detect the presence of PNAG in the sample. In one embodiment, the sample is a biological sample from a subject. The biological sample may be selected from the group consisting of urine, blood, pus, skin, sputum, joint fluid, lymph and milk. In one embodiment, the isolated binding agent is conjugated to a detectable label such as those described herein. A sample may also be derived from a swab of an implantable or implanted medical device.

Each of the limitations of the invention can encompass various embodiments of the invention. It is therefore anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

Brief Description of the Sequence Listing

SEQ ID NO:1 is the nucleotide sequence of the *ica* locus from S. aureus which has been deposited in GenBank under accession number AF086783.

Brief Description of the Figures

Fig. 1 shows the binding of antibody to native PNAG. The antibody was raised to native PNAG conjugated to diphtheria toxoid.

Fig. 2 shows binding of antibodies to deacetylated PNAG. The antibodies were raised to dPNAG conjugated to diphtheria toxoid.

Fig. 3 shows antibody titers obtained in mice (10 per group) immunized 3 times subcutaneously, one week apart, with native PNAG coupled to diphtheria toxoid (DTm). Animals were immunized with the dose indicated in the legend. Blood samples were obtained at weekly intervals 1-4 weeks after the final immunization.

Fig. 4 shows antibody titers obtained in mice (10 per group) immunized 3 times subcutaneously, one week apart, with dPNAG coupled to diphtheria toxoid (DTm). Animals were immunized with the dose indicated in the legend. Blood samples were obtained at weekly intervals 1-4 weeks after the final immunization.

Fig. 5 shows opsonic killing of *Staphylococcal* strains as indicated in the legend by antibodies from sera of a rabbit immunized with dPNAG conjugated to diphtheria toxoid (rabbit 1). Each point shows mean percentage killed at the indicated dilution.

Fig. 6 shows opsonic killing of *Staphylococcal* strains as indicated in the legend by antibodies from sera of a rabbit immunized with dPNAG conjugated to diphtheria toxoid (rabbit 2). Each point shows mean percentage killed at the indicated dilution.

Fig. 7 shows opsonic killing of *Staphylococcal* strains as indicated in the legend by antibodies from sera of a rabbit immunized with native PNAG conjugated to diphtheria toxoid (rabbit 3). Each point shows mean percentage killed at the indicated dilution.

Fig. 8 shows opsonic killing of *Staphylococcal* strains as indicated in the legend by antibodies from sera of a rabbit immunized with native PNAG conjugated to diphtheria toxoid (rabbit 4). Each point shows mean percentage killed at the indicated dilution.

Fig. 9 summarizes the opsonic killing titers of antibodies from sera of the four rabbits against the *Staphylococcal* strains indicated on X-axis. The rabbits are as described in the Figure legends above. Each bar shows the reciprocal of the serum dilution at which $\geq$ 40% of the bacteria were killed. Bars < 10 indicate sera unable to kill 40% of the bacteria at a 1:10 serum dilution.

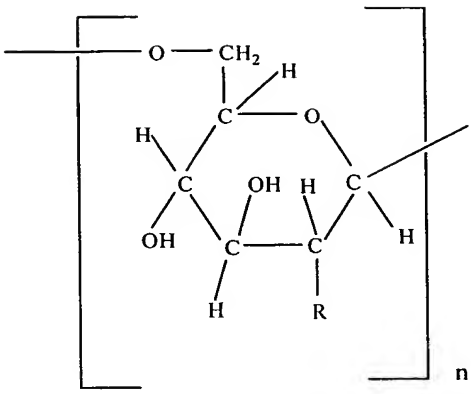
Detailed Description of the Invention

The invention relates to polysaccharide antigens derived from *Staphylococcal* bacteria. These antigens are useful for inducing immunity to bacterial infection and also for producing antibodies for diagnostic and therapeutic purposes.

The instant invention is based in part on the finding that poorly acetylated (i.e., deacetylated) poly-N-acetyl glucosamine (PNAG), referred to herein as dPNAG, is highly immunogenic and thus represents a suitable vaccine candidate for stimulating protective immune responses *in vivo*. A deacetylated PNAG is one having less than 50% of its amino groups substituted with acetate. In some preferred embodiments, there are 35% or fewer acetate substituents, while in others there are 15% or fewer acetate substituents. It has been further discovered, according to the invention, that dPNAG is better able to elicit opsonic protective antibodies than is native PNAG. "Native" PNAG refers to the naturally occurring mixture of PNAG with a range of acetylation levels ranging from 0-100%. dPNAG can be derived from native PNAG using the de-acetylation methods described herein. The antibodies prepared against dPNAG are thus effective against *Staphylococci* such as *S. aureus* and *S. epidermidis*. Accordingly, it has been discovered according to the invention that the extent of acetylation influences the level of immune response induced upon antigen administration *in vivo*. The antibodies elicited following dPNAG administration recognize dPNAG and in important embodiments also recognizes highly acetylated forms of PNAG.

The invention provides compositions of isolated dPNAG, methods of isolating and in some instances purifying dPNAG, as well as methods of use, including *in vivo* therapeutic, prophylactic and diagnostic methods. As used herein, the dPNAG may be referred to as dPNAG antigen. These latter terms are intended to be interchangeable. The invention also provides pharmaceutical compositions of dPNAG which may be used as vaccines.

In some aspects, dPNAG has the following structure:



where, n is an integer ranging from 2 to greater than or equal to 300, R is selected from the group consisting of -NH-CO-CH_3 and -NH_2, provided that less than 50% of the R groups are -NH-CO-CH_3. dPNAG has a beta (β) 1-6 linkage (i.e., it is comprised of glucosamine monomer units linked together by beta (β) 1-6 linkages).

dPNAG may be a homo-polymer if all the R groups are unsubstituted (i.e., R=NH_2). A homo-polymer is one in which the R groups of the glucosamine residues are identical. dPNAG can also be a hetero-polymer with a mixture of –NH_2 and –NH-CO-CH_3 groups at the R position provided that less than 50% of R groups are substituted with acetate. Depending on the embodiments, less than 49%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, or less than 1% of R groups may be substituted with acetate.

The size of dPNAG varies greatly, and depends upon whether dPNAG is conjugated to a carrier compound, as described herein. In some aspects, dPNAG antigen has a molecular weight of at least 100,000 Daltons. In other aspects, dPNAG antigen has a molecular weight of less than 2000 Daltons. The molecular weight of PNAG may be at least 200 Daltons, or at least 400 Daltons, or at least 600 Daltons, or at least 800 Daltons. Lower molecular weight dPNAG can be used according to the invention, preferably when conjugated to a carrier compound. These dPNAG can be as small as 2-3 monomer units, but preferably are at least 4-6 monomer units in length. The corresponding molecular weights for these are approximately 400, 600, 800, 1000 and 1200 Daltons. Polysaccharides between 500 and 20,000,000 Daltons will be typical.

As will be understood, the value of n in the above structure has an impact on the molecular weight of the antigen. If n is equal to or greater than 300, then the molecular weight of the minimal polysaccharide in the structure is 60,918 Daltons (300 units x 203

Daltons/unit + 18 Daltons for the substituents on the terminal residues). If the antigen has a minimum molecular weight of 100,000 Daltons, then either the polysaccharide has more than 300 units, or the polysaccharide is conjugated to a carrier compound which makes up for the difference in the molecular weight.

The invention provides both naturally occurring and synthetic forms of the dPNAG antigen. As used herein, the naturally occurring dPNAG is one that exists in or can be isolated or derived from naturally-occurring sources. dPNAG antigens are also provided in an isolated form. An isolated polysaccharide, such as isolated dPNAG, is one that has been removed and thus separated from the environment in which it normally exists. In some instances, an isolated polysaccharide is sufficiently separated from other compounds to be characterized structurally or functionally. For example, an isolated polysaccharide may be "sequenced" in order to determine its chemical composition.

dPNAG can be prepared from any bacterial strain carrying the *ica* locus. These strains include but are not limited to *S. epidermis* and *S. aureus,* and other strains (e.g., *S. carnosus*) that have been transformed with the genes in the *ica* locus. In particular, dPNAG can be prepared from specific strains including *S. epidermis* RP62A (ATCC number 35984), *S. epidermis* RP12 (ATCC number 35983), *S. epidermis* M187, *S. carnosus* TM300 (pCN27), *S. aureus* RN4220 (pCN27), and *S. aureus* MN8 mucoid.

One method involves incubating impure PNAG with a base or acid to produce a semi-pure PNAG preparation, neutralizing the preparation, and further treating the neutralized preparation to produce the dPNAG.

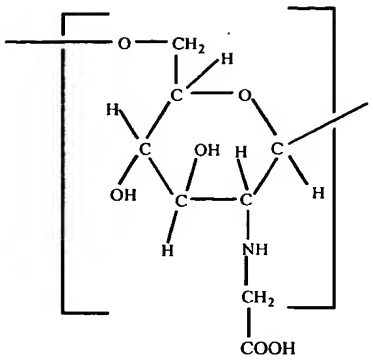
Impure native PNAG can be prepared by a variety of methods including extracting a crude native PNAG preparation from a bacterial culture, including cells and cell free culture supernatants, resulting in the isolation of a high molecular weight native PNAG-enriched material from the crude PNAG preparation, and obtained initially by precipitating an impure PNAG containing the high molecular weight PNAG-enriched material with a solvent such as methanol, ethanol, acetone or any other organic solvent known to one skilled in the art as being capable of causing the precipitation of polysaccharides from aqueous solutions. The steps of extracting the crude native PNAG preparation and isolating and precipitating the impure native PNAG antigen preparation are performed by any methods known in the art, such as those including U.S. Patent No. 5,055,455. This impure material is then purified and de-acetylated to produce dPNAG of the invention.

The purification steps are achieved by incubating impure PNAG with bacterial enzymes that can digest biological materials, including cell-wall disrupting agents such as lysozyme, lysostaphin, and proteinase K, and nuclease enzymes such as DNase and RNase to digest DNA and RNA. This is followed by an addition of a solvent that will precipitate PNAG out of solution, collection of the precipitate and re-dissolution of PNAG in a base, such as NaOH or an acid such as HCl, followed by neutralization. The neutralization can be accomplished using a base if the incubation step was performed with an acid, or with an acid if the incubation step was performed with a base. The insoluble fraction from the neutral material is then treated, e.g., by incubation in hydrofluoric acid to produce a pure native PNAG antigen or by re-dissolution in buffers with a pH < 4.0 followed by molecular sieve and/or ion-exchange chromatography.

Another isolation method includes the steps of extracting a crude PNAG suspension from a bacterial culture by incubating the bacteria with a strong base or acid. Preferably, the bacterial is stirred in the strong base or acid for at least 2 hours, and more preferably at least 5, 10, 15, 18 or 24 hours. The strong base or acid can be any type of strong base or acid, but preferably has a strength of at least 1 M NaOH or HCl. In some embodiments, the strong base or acid is 5 M NaOH or 5 M HCl. The acid or base solution is then subjected to centrifugation to collect the cell bodies. In some embodiments, the extraction procedure is repeated several times. The resultant acid or base solution is neutralized to approximately pH 7 and then dialyzed to produce insoluble impure PNAG.

dPNAG may be synthesized from naturally occurring polysaccharides that are greater than 50% acetate substituted. For instance, the dPNAG antigen may be synthesized by de-acetylating a heavily acetylated glucosamine polymer by chemical (e.g., base treatment) or by enzymatic means.

dPNAG antigens can also be synthesized *de novo*. (See, for example, Melean et al. Carbohydrate Research, 337:1893-1916, 2002.) Starting materials include, but are not limited to polyglucose (i.e., dextran), polyglucosamines, such as chitin or chitosan, and polyglucosaminouronic acid. Polygalactosaminouronic acid may also be used to produce the dPNAG antigen of the invention. Polyglucosamines having various substituents may also be modified to produce the PNAG antigen. For instance, polysaccharide intercellular adhesin (PIA) is a heavily acetylated polymer of ß-1-6 linked glucosamine residues. PIA has the following structure:



For those polysaccharides that contain imine moieties (C-NH), free amino groups can be formed by conventional chemistry techniques known to those of ordinary skill in the art. One suitable method involves the use of sodium borohydride. The imine group can be reduced with sodium borohydride to create a free amino group. This is done by adding in excess of 5 mg of borohydride to polysaccharide dissolved in distilled water while stirring at room temperature for 2 hours. The mixture is then dialyzed against water and freeze dried. (See, for example, DiFabio, et al. Biochem J., 1987 15; 244(1): 27-33).

The invention provides dPNAG preparations of varying purity. As used herein, a "pure dPNAG preparation" is a dPNAG preparation that has been isolated or synthesized and that is greater than 92% free of contaminants. These contaminants include heavily acetate substituted PNAG forms (i.e., greater than 50% acetate substitution), galactose, phosphate, teichoic acid, and the like. In some embodiments, dPNAG compositions are at least 93%, 94%, 95%, 96%, 97%, 98%, 99% free of contaminants or are 100% free of contaminants.

dPNAG compositions can also be referred to as "substantially free" of contaminants. A dPNAG composition substantially free of, for example, galactose indicates the presence of less than 10%, preferably less than 5%, or more preferably less than 1% galactose in a preparation containing dPNAG.

The degree of purity of the dPNAG composition can be assessed by any means known in the art. For example, the purity can be assessed by chemical analysis assays as well as gas chromatography and nuclear magnetic resonance to verify structural aspects of the material.

Another major contaminant of some dPNAG preparations can be phosphate-containing teichoic acid. The teichoic acid contamination can interfere with both the chemical characterization and the immunogenicity of the dPNAG antigen of the invention. The methods of the invention described herein are capable of producing an isolated dPNAG preparation that is substantially free of teichoic acid. A dPNAG preparation that is

substantially free of teichoic acid is one which has less than 1.0% phosphate, and more preferably one that has less than 0.1% phosphate. The amount of phosphate present in the sample can be assessed by any means known in the art. The amount of phosphate contamination can be assessed using the methods described in Keleti, G. and W.H. Lederer, ((1974) *Handbook of Micromethods for the Biological Sciences* Van Nostrand Reinhold Co., New York), which is hereby incorporated by reference. Briefly, the assay is performed as follows: to 100 μg of sample 100 μl of a solution made by adding together 43.5 ml of water, 6.5 ml of 70% perchloric acid ($HClO_4$) and 50 ml of 20 N sulfuric acid (H_2SO_4) is added. This is heated at 95°C for 2 hours in a tube with a marble on top of it. The mixture is then placed in an oven at 165°C and heated for an additional 2 hours, then cooled to room temperature. Next, one ml of reagent 5, made by the following method, is added to the sample:

Reagent 1: 1.36 grams of sodium acetate .$3H_20$ dissolved in 10 ml water.

Reagent 2: 500 mg ammonium molybdate dissolved in 20 ml water.

Reagent 3: 2 ml of reagent 1, 2 ml of reagent 2 and 16 ml of water.

Reagent 4: 2 gm ascorbic acid dissolved in 20 ml water, prepared immediately prior to use.

Reagent 5: Add in an ice bath 9 ml of reagent 3 and 1 ml of reagent 4.

After adding reagent 5 the tubes are mixed thoroughly and the optical density read at 820 nanometers in a spectrophotometer. A standard curve consisting of sodium phosphate monobasic (range of 0.1-5 μg per tube) is used to calculate the amount of phosphate present in the test samples. (Lowry, O.H., N.R. Roberts, K.Y. Leiner, M.L. Wu and A. L. Farr., (1954), *Biol. Chem.* 207, 1.)

The compositions of the invention are useful in a variety of different applications including *in vitro*, *in situ* and *in vivo* diagnosis of pathological status, such as infection. The compositions may be used to immunize subjects *in vivo* to prevent or treat infection. The compositions may also be used to develop antibodies and other binding peptides which are useful for the same purposes as the dPNAG compositions of the invention. Thus, the invention includes pharmaceutical compositions comprising dPNAG or corresponding binding agents (e.g., antibodies) that can be used for vaccination purposes to induce either active or passive immunity in a subject in need thereof. The invention also provides methods

for generating binding agents, such as antibodies that bind to dPNAG, which can be used in the diagnosis and treatment of *Staphylococcal* infections and associated conditions.

dPNAG may be used in a conjugated or an unconjugated form. In a conjugated form, dPNAG may be conjugated to a carrier compound, either directly or via a linker. The conjugation can occur at any position in the glucosamine monomer unit or at the ends of the polymer.

A "carrier compound" as used herein is a compound that can be conjugated to a polysaccharide either directly or through the use of a linker and that may be immunologically active or inert.

Carrier compounds include but are not limited to proteins, or peptides, polysaccharides, nucleic acids, or other polymers, lipids, and small molecules. Proteins include for example, plasma proteins such as serum albumin, immunoglobulins, apolipoproteins and transferrin; bacterial polypeptides such as TRPLE, β- galactosidase, polypeptides such as herpes gD protein, allergens, diphtheria and tetanus toxoids, salmonella flagellin, hemophilus pilin, hemophilus 15kDa, 28-30kDa and 40kDa membrane proteins, *Escherichia coli*, heat label enterotoxin ltb, cholera toxin, and viral proteins including rotavirus VP and respiratory syncytial virus f and g proteins. The proteins useful in the invention include any protein that is safe for administration to mammals and optionally that is an immunologically effective carrier protein.

Carrier compounds that are useful particularly for immunization include proteins such as keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soy bean trypsin inhibitor. Any other compound that is immunogenic in the species of animal to be immunized can similarly be used.

Many methods are known in the art for conjugating a polysaccharide to a protein. In general, the polysaccharide should be activated or otherwise rendered amenable to conjugation, i.e., at least one moiety must be rendered capable of covalently bonding to a protein or other molecule. Many such methods are known in the art. For instance, U.S. Patent No. 4,356,170, issued to Jennings, describes the use of periodic acid to generate aldehyde groups on the polysaccharide and then performs reductive amination using cyanoborohydride. U.S. Patent No. 4,663,160, issued to Tsay et al., also used periodic acid to generate aldehyde groups but then linked the polysaccharide to a protein derivatized with a 4-12 carbon moiety (prepared in the presence of a condensing agent) with a Schiff's base

reaction in the presence of a reducing agent such as cyanoborohydride. U.S. Patent No. 4,619,828, issued to Gordon, used cyanogen bromide to active the polysaccharide and then conjugated it through a spacer bridge of 4-8 carbon atoms to the protein. In U.S. Patent No. 4,808,700, issued to Anderson and Clements, a polysaccharide was modified to produce at least one reducing end using limited oxidative cleavage by periodate, hydrolysis by glycosidases, or acid hydrolysis and was conjugated to a protein through reductive amination in the presence of cyanoborohydride. U.S. Patent No. 4,711,779, issued to Porro and Costantino, described the activation of polysaccharides by introducing primary amino groups into the terminal reducing group using sodium cyanoborohydride, followed by conversion to esters in the presence of adipic acid derivatives and conjugation to a toxoid in the presence of an organic solvent, such as dimethylsulfoxide. Many other methods of conjugation are known in the art.

The carrier compound may be conjugated to dPNAG through a linker or spacer. A polysaccharide may be coupled to a linker or a spacer by any means known in the art including, for example using a free reducing end of the polysaccharide to produce a covalent bond with a spacer or linker. A covalent bond may be produced by converting a free reducing end of dPNAG into a free 1-aminoglycocide, that can subsequently be covalently linked to a spacer by acylation. (Lundquist et al., *J. Carbohydrate Chem.*, 10:377 (1991)). Alternatively, dPNAG may be covalently linked to the spacer using an N-hydroxysuccinimide active ester as activated group on the spacer. (Kochetkow, *Carbohydrate Research*, 146:C1 (1986)). The free reducing end of dPNAG may also be converted to a lactone using iodine and potassium hydroxide. (Isebell et al., *Methods of Carbohydrate Chemistry*, Academic Press, New York (1962)). The lactone can be covalently linked to the spacer by means of a primary amino group on the spacer or linker. The free reducing end of dPNAG may also be covalently linked to the linker or spacer using reductive amination.

The invention embraces antibodies that bind to dPNAG. The antibodies may be either monoclonal antibodies or polyclonal antibodies. The dPNAG antibodies bind to dPNAG and may also bind to forms of PNAG that are greater than 50% acetylated.

Polyclonal antibodies generally are raised in animals by multiple subcutaneous or intraperitoneal injections of an antigen and an adjuvant. Polyclonal antibodies to dPNAG can be generated by injecting dPNAG in conjugated or unconjugated form, alone or in combination with an adjuvant.

An example of polyclonal antibody preparation follows. dPNAG or a dPNAG conjugate is combined with an adjuvant such as Freund's complete adjuvant (e.g., 100 μg of conjugate for rabbits or mice in 1-3 volumes of Freund's) and injected intradermally at multiple sites. Approximately one month later, the animals are boosted with 1/5 - 1/10 of the original amount of antigen, or antigen conjugate, in adjuvant by subcutaneous injection at multiple sites. One to two weeks later the animals are bled, and the serum is assayed for the presence of antibody. The animals may be repeatedly boosted until the antibody titer plateaus. The animal may be boosted with dPNAG alone, dPNAG conjugate, or dPNAG conjugated to a different carrier compound, with or without an adjuvant. In some embodiments, the boosts may comprise PNAG rather than dPNAG, or they may contain a mixture of dPNAG and PNAG.

In addition to supplying a source of polyclonal antibodies, the immunized animals can be used to generate anti-dPNAG monoclonal antibodies. As used herein, the term "monoclonal antibody" refers to a homogenous population of immunoglobulins that bind to the same epitope (i.e., antigenic determinant) of dPNAG. This epitope may also be present in PNAG forms that are greater than 50% acetylated. Monoclonal antibodies have the same Ig gene rearrangement and thus demonstrate identical binding specificity. Monoclonal antibodies can be prepared by any method known in the art such as by immortalizing spleen cells isolated from the immunized animal by e.g., fusion with myeloma cells or by Epstein Barr Virus transformation, and screening for clones expressing the desired antibody. Other methods involve isolation of rearranged Ig gene sequences and cloning into immortalized cell lines. Methods for preparing and using monoclonal antibodies are well known in the art.

Murine anti-dPNAG monoclonal antibodies may be made by any of these methods utilizing dPNAG as an immunogen. The following description of a method for developing an anti-dPNAG monoclonal antibody is exemplary and is provided for illustrative purposes only. Balb/c mice are immunized intraperitoneally with approximately 75-100 μg of purified dPNAG in complete Freund's adjuvant. Booster injections of approximately 25-50 μg dPNAG in incomplete Freund's are administered on approximately days 15 and 35 after the initial injection. On day 60-65, the mice receive booster injections of approximately 25 μg dPNAG in the absence of adjuvant. Booster injection may alternatively comprise a native PNAG preparation or a mixture of dPNAG and PNAG. Three days later, the mice are killed and the isolated spleen cells fused to murine myeloma NS-1 cells using polyethylene glycol

by a procedure such as that described by Oi (Oi VT: Immunoglobulin-producing hybrid cell lines in *Herzenberg LA* (ed): Selected Methods in Cellular Biology, San Francisco, CA, Freeman, (1980)). Hybridoma cells are selected using hypoxanthine, aminopterin, and thymidine (HAT) and grown in culture. Fourteen to fifteen days after fusion, hybridoma cells producing anti-dPNAG monoclonal antibodies are identified using a solid-phase radioimmunoassay by capturing anti-dPNAG antibodies from conditioned media with immobilized goat anti-mouse IgG followed by quantitation of specifically bound ^{125}I-labeled dPNAG or PNAG. Hybridomas testing positive for antibodies against dPNAG are subcloned by limiting dilution and re-tested. Ascites for the hybridomas is then prepared in pristane-primed BALB/c mice by injecting approximately 1 x 10^6 cells/mouse. Concentrates enriched in the selected monoclonal antibodies are produced from ascites fluid by gel filtration on S-200 and concentrated with NH_4SO_4. The pellets are dissolved in an appropriate storage solution such as 50% glycerol/H_2O and are stored at 4°C.

An "anti-dPNAG antibody" as used herein includes humanized antibodies and antibody fragments as well as intact monoclonal and polyclonal antibodies that bind to dPNAG and in some instances to PNAG forms that are greater than 50% acetylated also. A "humanized monoclonal antibody" as used herein is a human monoclonal antibody or functionally active fragment thereof having at least human constant regions and a dPNAG binding region (e.g., a CDR) from a mammal of a species other than a human. An intact humanized anti-dPNAG monoclonal antibody in an isolated form or in a pharmaceutical preparation is particularly suited to some aspects of the invention. Humanized antibodies have particular clinical utility in that they specifically recognize dPNAG and preferably native PNAG forms also, but will not evoke an immune response in humans against the antibody itself. In one preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the " humanized antibody." See, e.g., L. Riechmann et al., Nature 332, 323 (1988); M. S. Neuberger et al., Nature 314, 268 (1985) and EPA 0 239 400 (published Sep. 30, 1987).

Human monoclonal antibodies may be made by any of the methods known in the art, such as those disclosed in US Patent No. 5,567,610, issued to Borrebaeck et al., US Patent No. 565,354, issued to Ostberg, US Patent No. 5,571,893, issued to Baker et al, Kozber, *J. Immunol.* 133: 3001 (1984), Brodeur, et al., *Monoclonal Antibody Production Techniques and Applications*, p. 51-63 (Marcel Dekker, Inc, new York, 1987), and Boerner et al., *J. Immunol.*,

147: 86-95 (1991). In addition to the conventional methods for preparing human monoclonal antibodies, such antibodies may also be prepared by immunizing transgenic animals that are capable of producing human antibodies (e.g., Jakobovits et al., *PNAS USA*, 90: 2551 (1993), Jakobovits et al., *Nature*, 362: 255-258 (1993), Bruggermann et al., *Year in Immunol.*, 7:33 (1993) and US Patent No. 5,569,825 issued to Lonberg).

The following examples of methods for preparing humanized monoclonal antibodies that interact with dPNAG and preferably other native PNAG forms also, are exemplary and are provided for illustrative purposes only. Humanized monoclonal antibodies, for example, may be constructed by replacing the non-CDR regions of a non-human mammalian antibody with similar regions of human antibodies while retaining the epitopic specificity of the original antibody. For example, non-human CDRs and optionally some of the framework regions may be covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. There are entities in the United States which will synthesize humanized antibodies from specific murine antibody regions commercially, such as Protein Design Labs (Mountain View California), Abgenix, and Medarex.

European Patent Application 0239400, the entire contents of which is hereby incorporated by reference, provides an exemplary teaching of the production and use of humanized monoclonal antibodies in which at least the CDR portion of a murine (or other non-human mammal) antibody is included in the humanized antibody. Briefly, the following methods are useful for constructing a humanized CDR monoclonal antibody including at least a portion of a mouse CDR. A first replicable expression vector including a suitable promoter operably linked to a DNA sequence encoding at least a variable domain of an Ig heavy or light chain and the variable domain comprising framework regions from a human antibody and a CDR region of a murine antibody is prepared. Optionally a second replicable expression vector is prepared which includes a suitable promoter operably linked to a DNA sequence encoding at least the variable domain of a complementary human Ig light or heavy chain respectively. A cell line is then transformed with the vectors. Preferably the cell line is an immortalized mammalian cell line of lymphoid origin, such as a myeloma, hybridoma, trioma, or quadroma cell line, or is a normal lymphoid cell which has been immortalized by transformation with a virus. The transformed cell line is then cultured under conditions known to those of skill in the art to produce the humanized antibody.

As set forth in European Patent Application 0239400 several techniques are well known in the art for creating the particular antibody domains to be inserted into the replicable vector. (Preferred vectors and recombinant techniques are discussed in greater detail below.) For example, the DNA sequence encoding the domain may be prepared by oligonucleotide synthesis. Alternatively a synthetic gene lacking the CDR regions in which four framework regions are fused together with suitable restriction sites at the junctions, such that double stranded synthetic or restricted subcloned CDR cassettes with sticky ends could be ligated at the junctions of the framework regions. Another method involves the preparation of the DNA sequence encoding the variable CDR containing domain by oligonucleotide site-directed mutagenesis. Each of these methods is well known in the art. Therefore, those skilled in the art may construct humanized antibodies containing a murine CDR region without destroying the specificity of the antibody for its epitope.

Human antibodies may also be obtained by recovering antibody-producing lymphocytes from the blood or other tissues of humans producing antibody to dPNAG. These lymphocytes can be treated to produce cells that grow on their own in the laboratory under appropriate culture conditions. The cell cultures can be screened for production of antibody to dPNAG and then cloned. Clonal cultures can be used to produce human monoclonal antibodies to dPNAG, or the genetic elements encoding the variable portions of the heavy and light chain of the antibody can be cloned and inserted into nucleic acid vectors for production of antibody of different types.

dPNAG binding antibody fragments are also encompassed by the invention. As is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions of the antibody, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. An isolated $F(ab')_2$ fragment is referred to as a bivalent monoclonal fragment because of its two antigen binding sites. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment,

retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd (heavy chain variable region). The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

The terms Fab, Fc, pFc', $F(ab')_2$ and Fv are employed with either standard immunological meanings [Klein, *Immunology* (John Wiley, New York, NY, 1982); Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* (Wiley & Sons, Inc., New York); Roitt, I. (1991) *Essential Immunology*, 7th Ed., (Blackwell Scientific Publications, Oxford)]. Well-known functionally active antibody fragments include but are not limited to $F(ab')_2$, Fab, Fv and Fd fragments of antibodies. These fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). For example, single-chain antibodies can be constructed in accordance with the methods described in U.S. Patent No. 4,946,778 to Ladner et al. Such single-chain antibodies include the variable regions of the light and heavy chains joined by a flexible linker moiety. Methods for obtaining a single domain antibody ("Fd") which comprises an isolated variable heavy chain single domain, also have been reported (see, for example, Ward et al., *Nature* 341:644-646 (1989), disclosing a method of screening to identify an antibody heavy chain variable region (V_H single domain antibody) with sufficient affinity for its target epitope to bind thereto in isolated form). Methods for making recombinant Fv fragments based on known antibody heavy chain and light chain variable region sequences are known in the art and have been described, e.g., Moore et al., US Patent No. 4,462,334. Other references describing the use and generation of antibody fragments include e.g., Fab fragments (Tijssen, Practice and Theory of Enzyme Immunoassays (Elsevieer, Amsterdam, 1985)), Fv fragments (Hochman et al., Biochemistry 12: 1130 (1973); Sharon et al., Biochemistry 15: 1591 (1976); Ehrilch et al., U.S. Patent No. 4,355,023) and portions of antibody molecules (Audilore-Hargreaves, U.S. patent No. 4,470,925). Thus, those skilled in the art may construct antibody fragments from various portions of intact antibodies without destroying the specificity of the antibodies for the dPNAG epitope. It is to be understood that the epitope recognized by anti-dPNAG antibodies may also be present on other native PNAG forms.

The antibody fragments also encompass "humanized antibody fragments." As one skilled in the art will recognize, such fragments could be prepared by traditional enzymatic cleavage of intact humanized antibodies. If, however, intact antibodies are not susceptible to such cleavage, because of the nature of the construction involved, the noted constructions can be prepared with immunoglobulin fragments used as the starting materials or, if recombinant techniques are used, the DNA sequences, themselves, can be tailored to encode the desired "fragment" which, when expressed, can be combined *in vivo* or *in vitro*, by chemical or biological means, to prepare the final desired intact immunoglobulin fragment.

Other dPNAG binding agents having binding specificity for dPNAG can be used in the diagnostic methods of the invention. Several routine assays may be used to easily identify dPNAG binding peptides. Screening assays for identifying peptides of the invention are performed for example, using phage display procedures such as those described in Hart, et al., *J. Biol. Chem.* 269:12468 (1994). Hart et al. report a filamentous phage display library for identifying novel peptide ligands for mammalian cell receptors. In general, phage display libraries using, e.g., M13 or fd phage, are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Ligands that bind selectively to dPNAG are obtained by selecting phage that express on their surface a ligand that binds to dPNAG. These phage then are subjected to several cycles of reselection to identify the peptide ligand-expressing phage that have the most useful binding characteristics. Typically, phage that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to dPNAG.

Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts.

To determine whether a peptide binds to dPNAG any known binding assay may be employed. For example, the peptide may be immobilized on a surface and then contacted with a labeled dPNAG. The amount of dPNAG which interacts with the peptide or the amount which does not bind to the peptide may then be quantitated to determine whether the

peptide binds to dPNAG. A surface having an anti-dPNAG antibody immobilized thereto may serve as a positive control. Binding assays may also determine the extent to which a putative dPNAG specific antibody binds to other native forms of PNAG.

The compositions of the invention are useful for many *in vivo,* and *in vitro* purposes. For example, the compositions of the invention are useful for producing an antibody response, e.g., as a vaccine for active immunization of humans and animals to prevent *Staphylococcal* infection and infections caused by other species of bacteria that make PNAG; as a vaccine for immunization of humans or animals to produce anti-dPNAG antibodies that can be administered to other humans or animals to prevent or treat *Staphylococcal* infections; as an antigen to screen for biological agents such as monoclonal antibodies capable of preventing *Staphylococcal* infection, libraries of genes involved in making antibodies, or peptide mimetics; as a diagnostic reagent for *Staphylococcal* infections and infections caused by other species of bacteria that make PNAG; and as a diagnostic reagent for determining the immunologic status of humans or animals in regard to their susceptibility to *Staphylococcal* infections and infections caused by other species of bacteria that make PNAG.

dPNAG can be used to protect a subject against infection with bacteria that make PNAG by inducing active immunity to infection by *Staphylococci* in a subject. The method is accomplished by administering to the subject an effective amount for inducing an immune response such as an antibody response against *Staphylococci* of any of the dPNAG compositions of the invention. "Active immunity" as used herein involves the introduction of an antigen into a subject such that the antigen causes differentiation of some lymphoid cells into cells that produce antibody and in certain instances other lymphoid cells into memory cells. The memory cells do not secrete antibodies but rather incorporate the antibodies into their membrane in order to sense antigen if it is administered to the body again.

The method is useful for inducing immunity to infection by *Staphylococci. "Staphylococci"* as used herein refers to all *Staphylococcal* bacterial species expressing the PNAG. Although not intending to be bound by any particular mechanism, it is thought that the highly acetylated forms of PNAG (i.e., > 50% acetylated) are not able to elicit production of opsonic, protective antibodies, to the same extent as dPNAG. Bacteria that are classified as *Staphylococci* are well known to those of skill in the art and are described in the microbiology literature. *Staphylococci* expressing PNAG include but are not limited *Staphylococcus epidermidis* (including RP62A (ATCC Number 35984), RP12 (ATCC Number 35983), and

M187), *Staphylococcus aureus* (including RN4220 (pCN27) and MN8 mucoid), and strains such as *Staphylococcus carnosus* transformed with the genes in the *ica* locus (including TM300 (pCN27)). Other bacterial strains expressing PNAG can be identified easily by those of ordinary skill in the art. For instance, *Staphylococcal* bacteria that express the *ica* locus will express PNAG. One of ordinary skill in the art can easily screen for the expression of mRNA or protein related to the *ica* locus since the nucleic acid sequence of the *ica* locus is known (SEQ ID NO:1 and originally described in Heilmann, C., O. Schweitzer, C. Gerke, N. Vanittanakom, D. Mack and F. Gotz (1996) Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis. Molec. Microbiol. 20:1083.*) Bacterial strains expressing PNAG also can be identified by immunoelectron microscopy (or other immunoassay) using anti-PNAG antibodies or anti-dPNAG antibodies to detect the presence of PNAG on the surface of the bacteria. Additionally the capsule of bacterial strains can be isolated and analyzed using liquid chromatography and mass spectroscopy.

A "subject" as used herein is a warm-blooded mammal and includes, for instance, humans, primates, horses, cows, swine, goats, sheep, dogs, and cats. In some embodiments, the subject is a non-rodent subject. A non-rodent subject is any subject as defined above, but specifically excluding rodents such as mice, rats, and rabbits. In some embodiments, the preferred subject is a human.

dPNAG may be administered to any subject capable of inducing an immune response such as an antibody response to an antigen. The antigen is especially suited to induce active immunization against systemic infection caused by *Staphylococci* in a subject capable of producing an immune response and at risk of developing a *Staphylococcal* infection. A subject capable of producing an immune response and at risk of developing a *Staphylococcal* infection is a mammal possessing an immune system that is at risk of being exposed to environmental *Staphylococci.* For instance, hospitalized patients are at risk of developing *Staphylococcal* infection as a result of exposure to the bacteria in the hospital environment. Particular high risk populations for developing infection by *S. aureus* include, for example, renal disease patients on dialysis, and individuals undergoing high risk surgery. High risk populations for developing infection by *S. epidermidis* also include, for example, patients with indwelling medical devices, such as intravenous lines (e.g., central lines), or prostheses (e.g., hip or knee replacement prostheses), because clinical isolates are often highly adherent to plastic surfaces due to their extracellular material (referred to as biofilm or slime). In some

embodiments, the subject is a subject that has received a medical device implant and in other embodiments, the subject is one that has not received a medical device implant but may be scheduled to receive one. Subjects at a high risk of developing infection by *S. epidermidis* further include, for example, pre-term neonates and patients undergoing chemotherapy.

dPNAG can be administered to the subject in an effective amount for inducing an antibody response. An "effective amount for inducing an immune response (e.g., an antibody response)" as used herein is an amount of dPNAG which is sufficient to (i) assist the subject in producing its own immune protection by e.g. inducing the production of anti-dPNAG antibodies in the subject (that may recognize both dPNAG and highly acetylated forms of PNAG), inducing the production of memory cells, and possibly a cytotoxic lymphocyte reaction etc. and/or (ii) prevent infection by *Staphylococci* from occurring in a subject which is exposed to *Staphylococci.*

In some preferred embodiments, the effective amount of a dPNAG vaccine for stimulating an immune response is an amount of dPNAG vaccine that is capable of eliciting the production of antibodies that are cross-reactive with at least two species of *Staphylococcus*, e.g., *S. aureus* and *S. epidermidis*.

One of ordinary skill in the art can assess whether an amount of dPNAG is sufficient to induce active immunity by routine methods known in the art. For instance, the ability of a specific antigen to produce antibody in a mammal can be assessed by screening for antibodies in a mouse or other subject using the dPNAG antigen.

The anti-dPNAG antibodies of the invention are useful for inducing passive immunization in a subject by preventing the development of systemic infection in those subjects at risk of exposure to infectious agents. The method for inducing passive immunity to infection by *Staphylococci* such as *Staphylococcus aureus* is performed by administering to a subject an effective amount of an anti-dPNAG antibody for inducing an immune response to *Staphylococci* e.g., by causing opsonization of *Staphylococci* such as *Staphylococcus aureus*. "Passive immunity" as used herein involves the administration of antibodies to a subject, wherein the antibodies are produced in a different subject (including subjects of the same and different species), such that the antibodies attach to the surface of the bacteria and cause the bacteria to be phagocytosed.

The anti-dPNAG antibody may be administered to any subject at risk of developing a *Staphylococcal* infection to induce passive immunity, and in some embodiments may be

particularly suited for subjects incapable of inducing active immunity to dPNAG. Since vaccination with dPNAG might not be completely effective in high risk immunocompromised subjects, these subjects will benefit from treatment with antibody preparations raised against *Staphylococci* such as *Staphylococcus aureus*. A subject that is incapable of inducing an immune response is an immunocompromised subject (e.g. patient undergoing chemotherapy, AIDS patient, etc.) or a subject that has not yet developed an immune system (e.g. pre-term neonate).

The anti-dPNAG antibody may be administered to a subject at risk of developing a *Staphylococcal* infection to prevent the infectious agent from multiplying in the body or to kill the infectious agent. The anti-PNAG antibody may also be administered to a subject who already has an infection caused by *Staphylococci* to prevent the infectious agent from multiplying in the body or to kill the infectious agent.

The anti-dPNAG antibody of the invention is administered to the subject in an effective amount for inducing an immune response to *Staphylococci* such as *Staphylococcus aureus*. An "effective amount for inducing an immune response to *Staphylococci*" as used herein is an amount of anti-dPNAG antibody that is sufficient to (i) prevent infection by *Staphylococci* from occurring in a subject which is exposed to *Staphylococci*; (ii) inhibit the development of infection, i.e., arresting or slowing its development; and/or (iii) relieve the infection, i.e., eradication of the bacteria in infected subjects.

Using routine procedures known to those of ordinary skill in the art, one can determine whether an amount of anti-dPNAG antibody is an "effective amount for inducing an immune response to *Staphylococci*" in an *in vitro* opsonization assay which is predictive of the degree of opsonization of an antibody. An antibody that opsonizes a *Staphylococcal* bacteria is one that when added to a sample of *Staphylococcal* bacteria causes phagocytosis of the bacteria. An opsonization assay may be a colorimetric assay, a chemiluminescent assay, a fluorescent or radiolabel uptake assay, a cell mediated bactericidal assay or other assay which measures the opsonic potential of a material. The following opsonization assay may be used to determine an effective amount of anti-dPNAG antibody. Anti-dPNAG antibody is incubated with an *Staphylococcal* bacteria and a eukaryotic phagocytic cell and optionally complement proteins. The opsonic ability of the anti-PNAG antibody is determined based on the amount of *Staphylococci* that remain after incubation. This can be accomplished by comparing the number of surviving *Staphylococci* between two similar assays, only one of

which includes opsonizing immunoglobulin. A reduction in the number of *Staphylococci*, as compared to incubation with control non-specific immunoglobulin, indicates opsonization.

The methods of the invention are also useful for inducing passive immunization to *Staphylococci* in a subject by administering to a subject an effective amount for inducing opsonization of *Staphylococci* of an anti-dPNAG$_{pure}$ antibody. An anti-dPNAG$_{pure}$ antibody as used herein is an antibody which specifically interacts with a pure dPNAG antigen of the invention and induces opsonization of coagulase-negative or coagulase-positive *Staphylococci* but that may not interact with an impure preparation of dPNAG. As discussed above, impure dPNAG preparations may be contaminated with teichoic acid or other impurities that can interfere with the immunogenicity of the antigen. One of ordinary skill in the art can easily identify whether an anti-dPNAG antibody is an anti-dPNAG$_{pure}$ antibody by using routine binding assays. For instance, an anti-dPNAG antibody may be immobilized on a surface and then contacted with a labeled impure dPNAG preparation or a labeled pure dPNAG preparation. The amount of dPNAG preparation (pure vs. impure preparation) which interacts with the antibody or the amount which does not bind to the antibody may then be quantitated to determine whether the antibody binds to an impure dPNAG preparation. In important embodiments, the anti- dPNAG$_{pure}$ antibody is effective against coagulase-negative and coagulase-positive *Staphylococci* or against any appropriate microbial organism expressing dPNAG or highly acetylated PNAG on its surface.

dPNAG antigen may be formulated as a vaccine. A suitable carrier media for formulating a vaccine includes sodium phosphate-buffered saline (pH 7.4) or 0.125 M aluminum phosphate gel suspended in sodium phosphate-buffered saline at pH 6 and other conventional media. Generally, vaccines contain from about 5 to about 100 μg, and preferably about 10-50 μg of the antigen to elicit effective levels of antibody in warm-blooded mammals. When administered as a vaccine the dPNAG can optionally include an adjuvant.

The term "adjuvant" is intended to include any substance which is incorporated into or administered simultaneously with dPNAG, which potentiates the immune response in the subject. Adjuvants include but are not limited to aluminum compounds, e.g., gels, aluminum hydroxide and aluminum phosphate, and Freund's complete or incomplete adjuvant (e.g., in which the dPNAG antigen is incorporated in the aqueous phase of a stabilized water in paraffin oil emulsion). The paraffin oil may be replaced with different types of oils, e.g., squalene or peanut oil. Other materials with adjuvant properties include BCG (attenuated

Mycobacterium tuberculosis), calcium phosphate, levamisole, isoprinosine, polyanions (e.g., poly A:U), lentinan, pertussis toxin, lipid A, saponins, QS-21 and peptides, e.g. muramyl dipeptide. Rare earth salts, e.g., lanthanum and cerium, may also be used as adjuvants. The amount of adjuvants depends on the subject and the particular dPNAG antigen used (e.g., the level of acetate substitution) and can be readily determined by one skilled in the art without undue experimentation.

In general, when administered for therapeutic purposes, the formulations of the invention are applied in pharmaceutically acceptable solutions. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

The compositions of the invention may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicyclic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% W/V); citric acid and a salt (1-3% W/V); boric acid and a salt (0.5-2.5% W/V); and phosphoric acid and a salt (0.8-2% W/V). Suitable preservatives include benzalkonium chloride (0.003-0.03% W/V); chlorobutanol (0.3-0.9% W/V); parabens (0.01-0.25% W/V) and thimerosal (0.004-0.02% W/V).

The present invention provides pharmaceutical compositions, for medical use, that comprise dPNAG together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The term "pharmaceutically-acceptable carrier" as used herein, and described more fully below, means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other animal. In the present invention, the term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the

application. The components of the pharmaceutical compositions also are capable of being commingled with dPNAG, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the polysaccharide, which can be isotonic with the blood of the recipient. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for subcutaneous, intramuscular, intraperitoneal, intravenous, etc. administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

The preparations of the invention are administered in effective amounts. An effective amount, as discussed above, is that amount of dPNAG or anti-dPNAG antibody that will alone, or together with further doses, induce active immunity or opsonization of the infectious bacteria, respectively. It is believed that doses ranging from 1 nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, will be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms/kilogram, and most preferably between 1 microgram and 100 micrograms/kilogram. The absolute amount will depend upon a variety of factors including whether the administration is performed on a high risk subject not yet infected with the bacteria or on a subject already having an infection, the concurrent treatment, the number of doses and the individual patient parameters including age, physical condition, size and weight. These are factors well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment.

Multiple doses of the pharmaceutical compositions of the invention are contemplated. Generally immunization schemes involve the administration of a high dose of an antigen followed by subsequent lower doses of antigen after a waiting period of several weeks. Further doses may be administered as well. The dosage schedule for passive immunization would be quite different with more frequent administration if necessary. Any regimen that results in an enhanced immune response to bacterial infection and/or subsequent protection

from infection may be used. Desired time intervals for delivery of multiple doses of a particular dPNAG can be determined by one of ordinary skill in the art employing no more than routine experimentation.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular dPNAG selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, intraperitoneal, and intrasternal injection, or infusion techniques. Other routes include but are not limited to oral, nasal, dermal, sublingual, and local.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing dPNAG or a dPNAG binding agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the polymer into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. The polymer may be stored lyophilized.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the polysaccharides of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer based systems such as polylactic and polyglycolic acid, polyanhydrides and polycaprolactone; nonpolymer systems that are lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di and triglycerides; hydrogel release systems; silastic systems; peptide based systems; wax coatings, compressed tablets using conventional binders and excipients, partially fused implants and the like. Specific examples include, but are not limited to: (a) erosional systems in which the polysaccharide is contained in a form within a matrix, found in U.S. Patent Nos. 4,452,775 (Kent); 4,667,014 (Nestor et al.); and 4,748,034 and 5,239,660 (Leonard) and (b) diffusional systems in which an active component permeates at a controlled rate through a polymer, found in U.S. Patent

Nos. 3,832,253 (Higuchi et al.) and 3,854,480 (Zaffaroni). In addition, a pump-based hardware delivery system can be used, some of which are adapted for implantation.

It will also be appreciated by those of ordinary skill in the art that the PNAG antigens of the present invention may have adjuvant properties by themselves. To the extent that the polysaccharides described herein potentiate human immune responses, they can be used as adjuvants in combination with other materials.

The dPNAG antigens and anti-dPNAG antibodies of the invention may be delivered in conjunction with another anti-bacterial (i.e., bactericidal) drug or in the form of anti-bacterial cocktails or with other bacterial antigens or anti-bacterial antibodies. An anti-bacterial antibiotic cocktail is a mixture of any of the compositions of the invention with an anti-bacterial drug. The use of antibiotics in the treatment of bacterial infection is routine. The use of antigens for inducing active immunization and antibodies to induce passive immunization is also routine. In this embodiment, a common administration vehicle (e.g., tablet, implant, injectable solution, etc.) could contain both the composition useful in this invention and the anti-bacterial antibiotic drug and/or antigen or antibody. Alternatively, the anti-bacterial antibiotic drug and/or antigen or antibody can be separately dosed. The anti-bacterial agent (e.g., an antibiotic) can also be conjugated to dPNAG or to an anti-dPNAG antibody.

Anti-bacterial antibiotic drugs are well known and include: penicillin G, penicillin V, ampicillin, amoxicillin, bacampicillin, cyclacillin, epicillin, hetacillin, pivampicillin, methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin, carbenicillin, ticarcillin, avlocillin, mezlocillin, piperacillin, amdinocillin, cephalexin, cephradine, cefadoxil, cefaclor, cefazolin, cefuroxime axetil, cefamandole, cefonicid, cefoxitin, cefotaxime, ceftizoxime, cefmenoxine, ceftriaxone, moxalactam, cefotetan, cefoperazone, ceftazidme, imipenem, clavulanate, timentin, sulbactam, neomycin, erythromycin, metronidazole, chloramphenicol, clindamycin, lincomycin, vancomycin, trimethoprim-sulfamethoxazole, aminoglycosides, quinolones, tetracyclines and rifampin. (See Goodman and Gilman's, Pharmacological Basics of Therapeutics, 8th Ed., 1993, McGraw Hill Inc.)

Other polysaccharide antigens and antibodies are well known in the art. For instance, the following polysaccharide antigens and/or antibodies thereto can be administered in conjunction with the dPNAG antigen and/or antibody: *Salmonella typhi* capsule Vi antigen (Szu, S.C., X. Li, A.L. Stone and J.B. Robbins, Relation between structure and immunologic

properties of the Vi capsular polysaccharide, *Infection and Immunity.* 59:4555-4561 (1991)); *E. Coli* K5 capsule (Vann, W., M.A. Schmidt, B. Jann and K. Jann, The structure of the capsular polysaccharide (K5 antigen) of urinary tract infective *Escherichia coli*, 010:K5:H4. A polymer similar to desulfo-heparin, *European Journal of Biochemistry.* 116: 359-364, (1981)); *Staphylococcus aureus* type 5 capsule (Fournier, J.-M., K. Hannon, M. Moreau, W.W. Karakawa and W.F. Vann, Isolation of type 5 capsular polysaccharide from *Staphylococcus aureus, Ann. Inst. Pasteur/Microbiol.* (Paris). 138: 561-567, (1987)); *Rhizobium melilori* expolysaccharide II (Glazebrook, J. and G.C. Walker, a novel expolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti, Cell.* 65:661-672 (1989)); *Group B streptococcus* type III (Wessels, M.R., V. Pozsgay, D.L. Kasper and H. J. Jennings, Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III *group B Streptococcus, Journal of Biological Chemistry.* 262:8262-8267 (1987)); *Pseudomonas aeruginosa* Fisher 7 O-specific side-chain (Knirel, Y.A., N.A. Paramonov, E.V. Vinogradov, A.S. Shashkow, B.A. N.K. Kochetkov, E.S. Stanislavsky and E.V. Kholodkova, Somatic antigens of *Pseudomonas aeruginosa* The structure of O-specific polysaccharide chains of lipopolysaccharides of *P. aeruginosa* O3 (Lanyi), 025 (Wokatsch) and Fisher immunotypes 3 and 7, *European Journal of Biochemistry.* 167:549, (1987)); *Shigella sonnei* O-specific side chain (Kenne, L., B. Lindberg and K. Petersson, Structural studies of the O-specific side-chains of the *Shigella sonnei* phase I lipopolysaccharide, *Carbohydrate Research.* 78:119-126, (1980)); *S. pneumoniae* type I capsule (Lindberg, B., Lindqvist, B., Lonngren, J., Powell, D.A., Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 1, *Carbohydrate Research.* 78:111-117 (1980)); and *Streptococcus pneumoniae* group antigen (Jennings, H.J., C. Lugowski and N. M. Young, Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type 1, *Biochemistry.* 19:4712-4719 (1980)).

Other non-polypeptide antigens and antibodies thereto are well known to the those of skill in the art and can be used in conjunction with the dPNAG compositions of the invention.

The dPNAG antigens and antibodies are also useful in diagnostic assays for determining an immunologic status of a subject or sample or can be used as reagents in immunoassays. For instance, the antibodies may be used to detect the presence in a sample of a bacteria having PNAG on the surface. If the bacteria is present in the sample, then the

antibodies may be used to treat the infected subject. The antibodies may also be used to screen bacteria for the presence of PNAG antigen and to isolate dPNAG or PNAG antigen and bacteria containing dPNAG or PNAG antigen from complex mixtures.

The above-described assays and any other assay known in the art can be accomplished by labeling the dPNAG or antibodies and/or immobilizing the dPNAG or antibodies on an insoluble matrix. The analytical and diagnostic methods for using dPNAG and/or its antibodies use at least one of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner, and steric conjugates. The label used can be any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for such use in immunoassays. For example, compounds that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as compounds that can be detected through reaction or derivitization, such as enzymes. Examples of these types of labels include ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I radioisotopes, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases such as firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalavinediones, horseradish peroxidase (HRP), alkaline phosphatase, ß-galactosidase, glucoamylase, lysozyme, saccharide oxidases such as glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase. Heterocyclic oxidases such as uricase and xanthine oxidase, coupled to an enzyme that uses hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin avidin, spin labels, bacteriophage labels, and stable free radicals.

The labels can be conjugated to dPNAG or anti-dPNAG antibody by methods known to those of ordinary skill in the art. For example, U.S. Patent Nos. 3,940,475 and 3,645,090 demonstrate conjugation of fluorophores and enzymes to antibodies. Other assays which reportedly are commonly used with antigen and antibody and which can be used according to the invention include competition and sandwich assays.

The invention includes a method of preparing dPNAG antigen by producing a PNAG expressing host cell, by introducing an *ica* locus into a cell, isolating PNAG antigen from such a cell, and de-acetylating the antigen to form dPNAG. A PNAG host cell can be prepared by transfecting transducing or transforming a cell with the nucleic acid encoding the

ica gene (SEQ ID NO:1). The cell can be a eukaryotic or prokaryotic cell but preferably is a bacterial cell. The cell may be a *Staphylococci* that does not naturally express PNAG.

The *ica* nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the *ica* nucleic acid within a eukaryotic or prokaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the *ica* nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, and ß-actin. Exemplary viral promoters which function constitutively in cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively. Such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined *ica* nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

The *ica* nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the *ica* coding sequence under the influence or control of the gene expression sequence. If it is desired that the *ica* sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the *ica* sequence and if the nature of

the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the *ica* sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a *ica* nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that *ica* nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The *ica* nucleic acid of the invention can be delivered to the host cell alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a nucleic acid molecule containing the genes in the *ica* locus that encode proteins involved in PNAG synthesis or (2) uptake of a nucleic acid molecule containing the genes in the *ica* locus that encode proteins involved in PNAG synthesis by a target cell. Preferably, the vectors transport the *ica* molecule into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are useful for delivery/uptake of *ica* nucleic acids to/by a target cell. Chemical/physical vectors are useful for delivery/uptake of *ica* nucleic acids or *ica* polypeptides to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be attached to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as: Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes viruses; vaccinia viruses; polio viruses; and RNA viruses such as any retrovirus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses

are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman Co., New York (1990) and Murry, E.J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Cliffton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication - deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for at least 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

In addition to the biological vectors, chemical/physical vectors may be used to deliver a *ica* molecule to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the *ica* molecule to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector in vivo or in vitro. It has been shown that large unilamellar vessels (LUV), which range in size from 0.2 - 4.0 μm can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, (1981) 6:77). In order for a liposome to be an

efficient gene transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the gene of interest at high efficiency with retention of biological activity; (2) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (3) accurate and effective expression of genetic information.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, (1985) 3:235-241.

Compaction agents also can be used alone, or in combination with, a biological or chemical/physical vector of the invention. A "compaction agent", as used herein, refers to an agent, such as a histone, that neutralizes the negative charges on the nucleic acid and thereby permits compaction of the nucleic acid into a fine granule. Compaction of the nucleic acid facilitates the uptake of the nucleic acid by the target cell. The compaction agents can be used alone, i.e., to deliver the *ica* molecule in a form that is more efficiently taken up by the cell or, more preferably, in combination with one or more of the above-described vectors.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the *ica* nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a *ica* nucleic acid into a preselected location within the target cell chromosome).

The following examples are included for purposes of illustration and are not intended to limit the scope of the invention.

Examples

Example 1: Purification of dPNAG.

It has been discovered according to the invention that dPNAG can be produced from any bacterial strain expressing the *ica* locus. Specifically, these include *Staphylococcus epidermidis*, *Staphylococcus aureus*, and other *Staphylococcal* strains such as *Staphylococcus carnosus* transformed with the genes in the *ica* locus. The following specific strains can be

used according to the invention to purify PNAG from include *S. epidermidis* RP62A (ATCC Number 35984), *S. epidermidis* RP12 (ATCC Number 35983), *Staphylococcus epidermidis* M187, *S. carnosus* TM300 (pCN27), *S. aureus* RN4220 (pCN27), and S. *aureus* MN8 mucoid.

The following is a method that can be used for producing dPNAG from *Staphylococci* containing the *ica* locus.

Starting material is prepared from cultures of *Staphylococci* expressing the *ica* genes by growing the bacteria as follows: The polysaccharide is prepared from 16 liter cultures of bacterial growth medium. A preferred medium is a chemically-defined medium (CDM) based upon RPMI-1640 AUTO-MOD, a preparation of RPMI modified to allow sterilization by autoclaving (Sigma Chemical Co., St, Mo.). The CDM is supplemented with additional amino acids, vitamins and nucleotides to adjust their concentration to those found in other CDM (Hussain, M., J.G.M. Hastings, and P.J. White, 1991). A chemically defined medium for slime production by coagulase-negative *Staphylococci*. J. Med. Microbiol. 34:143-147. The medium is also supplemented with sucrose and glucose to a final concentration of 1%.

Liquid cultures are inoculated with a single colony of a polysaccharide-producing strain of bacteria. The preferred strain is designated *Staphylococcus aureus* MN8m, a strain that is a constitutive over-producer of the polysaccharide. A single colony is taken from a tryptic soy agar plate, or similar plate of bacterial growth medium, and grown at 37°C. Temperatures of 10-42°C are also acceptable. Liquid cultures are incubated at 37°C for 1-96 hours while being continuously stirred and flushed with oxygen at a rate of 2 liters/min. The pH is maintained at 7.0 throughout the growth period by the addition of 10 N NaOH via a pH titrator. At the end of the growth period, cell bodies are sedimented at 9000 g for 30 minutes and the supernatant concentrated to ~500 ml via tangential-flow filtration (10,000-500,000 molecular weight cutoff membranes). Two volumes of ethanol are added to precipitate the crude polysaccharide preparation. The precipitate is recovered by centrifugation, re-suspension in water and overnight dialysis against distilled water. The antigen is insoluble. The insoluble, crude antigen is suspended in 50 ml of phosphate buffered saline (PBS, 0.1 M phosphate, 0.15 M sodium chloride) to be digested with the lysozyme (0.5mg) and lysostaphin (0.5 mg) for 0.5 to 16 h at 37°C. Antigen suspensions are further treated with nucleases (0.5 mg) at 37°C for 0.5 to 16 h followed by incubation for 0.5 to 16 h with proteinase K (5 mg) at 37-56°C. After dialysis and lyophilization, dried extracts are dissolved

in 5 M HCl and the pH adjusted to 2 with 4 N NaOH. Twenty ml aliquots of this solution are applied to a 5x88 cm column packed with Sephacryl S-300 (Pharmacia, Piscataway, NJ) using 0.1 N HCl/0.15 M NaCl buffer with the eluted polysaccharide identified by optical absorption at 206 nm. Fractions corresponding to the polysaccharide representing a continuous range of molecular sizes are separately pooled, dialyzed against water, and lyophilized. Alternately, size fractionation can be performed with a variety of alternative procedures known in the art such as use of diafiltration membranes.

The level of acetylation can be adjusted by chemically-treating the native polysaccharide. Thus, polysaccharide with > 50 % acetate is isolated, and de-acetylated to achieve the desired acetylated level. Treatment is in a basic solution known to remove amino-linked acetate groups from glucosamine. A preferred means is incubation at 37°C for 2-20 hours in 1.0 M NaOH. Weaker solutions and longer incubation times or higher temperatures, or stronger solutions with shorter incubation times or lower temperatures are equally effective. Generally, any treatment that raises the pH above 10 would be effective under the proper temperature.

There are also enzymatic means to de-acetylate the antigen. These include de-acetylating enzymes such as those related to chloroamphenicol de-acetylase and the icaB gene product.

Example 2: Preparation of dPNAG Diphtheria Toxoid (DTm) Conjugate Vaccine.

DTm was covalently coupled to purified dPNAG by reductive amination. Aldehyde groups were first introduced onto the surface of diphtheria toxoid (DTm) by treatment of the protein with glutaraldehyde as described in step 1 below. Activated DTm was subsequently reacted with dPNAG, through its free amino groups in the presence of the reducing agent sodium cyanoborohydride as described in step 2 below.

Step 1: Activation of DTm with glutaraldehyde

10 mg of DTm (4.86 mg/ml solution in 20 mM HEPES buffer, 50 mM NaCl, pH 8) were dialyzed against 0.1 M carbonate buffer (pH 10) for 3 hours (h) at room temperature using a 10 kDa MWCO dialysis cassette. When the protein solution was completely exchanged with carbonate buffer, glutaraldehyde was added to a final concentration of 1.25 % and the mixture stirred at room temperature for 2h. This produced activated DTm, which was

exchanged with Phosphate Buffer Saline (PBS, pH 7.4) and concentrated to approximately 10 mg/ml by ultrafiltration using a 10 kDa MWCO filtration membrane.

Step 2: Coupling of activated-DTm to PNAG

PNAG was purified as described in Maira et al. (Maira-Litrán T, Kropec A, Abeygunawardana C, Joyce J, Mark III G, Goldmann DA, and Pier GB. Immunochemical properties of the staphylococcal poly-N-acetyl glucosamine surface polysaccharide. Infect. Immun. 2002; 70:4433-4440). One fraction of this material, designated PNAG-II in Maira et al., was used to prepare the deacetylated PNAG (dPNAG). Native PNAG was dissolved to a concentration of 2 mg/ml in 5 M NaOH and incubated at 37°C with stirring. After 18 h, the sample was placed in an ice slurry and allowed to cool to $\leq$10°C. 5 N HCl was also cooled on ice and added in 0.5 mL aliquots until the solution reached neutral pH. The dPNAG solution was then dialyzed overnight against distilled water in a 10 KiloDalton Molecular Weight Cutoff (10K MWCO) dialysis cassette and lyophilized. This procedure yielded dPNAG having 15-20% of acetate substitutions.

Purified dPNAG (10 mg) was dissolved in 0.25 ml of 5 M HCl, neutralized with an equal volume of 5 M NaOH and the final volume adjusted to 2ml with PBS. dPNAG solutions are insoluble at neutral pH but remain completely soluble at slightly acidic or basic pH. Therefore to ensure solubility, the pH of dPNAG solutions was adjusted to 9.0. dPNAG (10mg) was mixed with 1 ml of a 10 mg/ml solution of activated DTm in PBS and pH of the reaction adjusted to 7.5. Two hundred mg of purified sodium cyanoborohydride was added to the mixture and the reaction allowed to proceed in the dark for 14 h at 37°C with mixing. After this time, the reaction mixture was exchanged by dialysis with 0.1 M carbonate buffer, 0.15 M NaCl, pH 10 (10 kDa MWCO dialysis cassette) and the high molecular weight conjugate was purified away from uncoupled components with a Superose 6 prep-grade column by gel filtration chromatography. dPNAG-DTm conjugate was dialyzed against 20 mM HEPES buffer, 50 mM NaCl, pH 8 and stored frozen at –2°C.

Example 3: Preparation of Native PNAG-DTm Conjugate Vaccine.

Native PNAG (in this case, having 95% - 100% acetate substitutions) was covalently coupled to purified DTm using the organic cyanylating agent 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) to activate the polysaccharide hydroxyl

groups as described in Step 1 below. CDAP-activated PNAG was subsequently coupled to DTm as described in Step 2 below without the need for additional spacer molecules.

Step 1: Activation of PNAG with CDAP

10 mg of purified PNAG were dissolved in 150 microliters of 5 M HCl, neutralized with an equal volume of 5 M NaOH and diluted up to 1 ml with borate buffer pH 9.2. CDAP was made up at 100 mg/ml concentration in acetonitrile and stored at –20°C for up to 1 month. 200 microliters of CDAP (containing 20 mg) were slowly pipetted into a previously vortexed solution of PNAG-II (Maira, et al. Infect. Immun. 2002, 70: 4433-4440) in borate buffer (rapid addition of the organic co-solvent precipitates the polysaccharide) and the reaction was allowed to proceed for two minutes.

Step 2: Coupling of CDAP-Activated PNAG with DTm

5 mg of DTm (stock solution in 20 mM HEPES buffer, 50 mM NaCl, pH 8) were dialyzed against borate buffer pH 9.2 for 3h with a 10 kDa MWCO dialysis cassette. After the activation of PNAG with CDAP, 5 mg of DTm was immediately added and the mixture reacted at room temperature for 3h with stirring. After this time, the high molecular weight conjugate was purified from uncoupled components with a Superose 6 prep-grade column by gel filtration chromatography. Fractions containing PNAG-DTm conjugate were pooled, concentrated and stored frozen at –20°C.

Example 4: Production of Antiserum in Rabbits.

Antibodies to purified PNAG-DTm or to dPNAG-DTm were raised in New Zealand white rabbits by subcutaneous immunization with two 10 μg doses of conjugated polysaccharide emulsified for the first dose in complete Freund's adjuvant and for the second dose in incomplete Freund's adjuvant, followed one week later by three intravenous injections of antigen in saline, each spaced three days apart. Rabbits were bled every two weeks and sera tested by ELISA. Binding curves obtained by ELISA from two representative rabbits immunized with either PNAG or dPNAG-DTm conjugates are shown in Figs. 1 and 2, respectively. Titers were determined as described by Maira et al. (Maira-Litrán T, Kropec A, Abeygunawardana C, Joyce J, Mark III G, Goldmann DA, and Pier GB. Immunochemical properties of the *staphylococcal* poly-N-acetyl glucosamine surface polysaccharide. Infect. Immun. 2002; 70:4433-4440).

Example 5: Immunogenicity of PNAG-DTm and dPNAG-DTm in Mice.

Groups of ten mice (Swiss Webster; female, 5-7 weeks of age) were immunized subcutaneously, one week apart, with 1.5, 0.75 or 0.15 μg of conjugated polysaccharide of PNAG-DTm and dPNAG-DTm in 0.1 ml of PBS and bled weekly for four weeks after the 3rd immunization. Control groups were immunized with a mixture of unconjugated polysaccharide and protein in the same ratio. Titers of mice immunized with the native and de-acetylated conjugates are shown in Figs. 3 and 4, respectively. Control groups developed no titers at any on the doses used.

Example 6: Opsonic Killing Activity of Rabbit Antisera Raised to PNAG and dPNAG Conjugated to Tetanus Toxoid.

Two rabbits were immunized with PNAG conjugated to diphtheria toxoid and two rabbits were immunized with dPNAG conjugated to diphtheria toxoid as described above. Opsonic killing activity was determined using the method described by Maira et al. (Maira-Litrán T, Kropec A, Abeygunawardana C, Joyce J, Mark III G, Goldmann DA, and Pier GB. Immunochemical properties of the *Staphylococcal* poly-N-acetyl glucosamine surface polysaccharide. Infect. Immun. 2002; 70:4433-4440). The titer was determined, and defined as the serum dilution at which ≥ 40 % of the bacteria were killed. Binding curves of the 4 rabbit antisera against a variety of *Staphylococcal* strains is shown in Figs. 5-8. Strain M187 is a *S. epidermidis* strain; the others are all *S. aureus* strains. Titer comparisons are shown in Fig. 9.

Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

We claim: